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COMPONENTS OF HONEYBEE ROYAL JELLY AS DETERRENTS OF THE PARASITIC *Varroa* MITE, *Varroa destructor*

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Abstract—The parasitic mite *Varroa destructor* Anderson & Trueman reproduces on the immature stage of the honeybee, *Apis mellifera* L. Mites are found more often on drone brood than worker brood and only infrequently on queen brood. We investigated the chemical basis for the low incidence of mites on queen brood. *V. destructor* mites were deterred by a crude extract of royal jelly, a glandular secretion produced by nurse bees and fed to queen larvae. Bioassay-driven fractionation of the crude extract via column chromatography resulted in one active fraction that was as active as the crude extract. Compounds in the active fraction were identified using gas chromatography (GC) and coupled gas chromatography/mass spectrometry (GC-MS). Before injection, compounds were esterified with MeOH/sulfuric acid, followed by silylation of any hydroxyl groups present. The active fraction contained at least 22 compounds, all fatty acids, several of which contained an additional hydroxyl group on the alkyl chain. Synthesis of some of these compounds that are not commercially available is described. A synthetic mixture containing most of the compounds in the active fraction was as active as the active fraction in the bioassay.

Key Words V Honey bee, host location, royal jelly, parasitic mite, deterrent, *Apis mellifera*, *Varroa destructor*.

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INTRODUCTION

Varroa destructor Anderson & Trueman is a parasitic mite of the honeybee *A. mellifera* L. and the greatest threat to beekeeping worldwide. Mites require the immature stage of their host for reproduction (reviewed in De Jong et al., 1982; De Jong, 1990). Those reproducing on drone brood (males) average 2.2Y2.6 female offspring per host, whereas those reproducing on worker brood (females) average 1.3Y1.4 female offspring per host (Schulz, 1984; Fuchs and Langenbach, 1989). Mites do not reproduce on queen brood (also females) (Romaniuk et al., 1988; Rehm and Ritter, 1989; Harizanis, 1991; Santillan-Galicia et al., 2002). The rate of infection of immature honeybees varies with their sex and caste, reflecting the opportunity each type of host affords the mite for reproduction. Mites are found more often on drone brood than worker brood, with average differences ranging between 5- and 12-fold (Grobov, 1977; Sulimanovic et al., 1982; Schulz, 1984; Issa and Goncalves, 1984; Fuchs, 1990, 1992; Boot et al., 1991; Calderone and Kuenen, 2001). Mites are found infrequently on queen brood (Harizanis, 1991; Calderone et al., 2002; Santillan-Galicia et al., 2002).

Numerous studies have sought to identify the mechanisms responsible for the different rates at which worker and drone brood are parasitized. Both chemical and physical factors appear to be involved (De Jong and Morse, 1988; Le Conte et al., 1989; Rickli et al., 1992, 1994; Goetz and Koeniger, 1993; Donzé et al., 1998; Kuenen and Calderone, 1998, 2000; Beetsma et al., 1999; Calderone and Kuenen, 2003; Calderone and Lin, 2001; Nazzi et al., 2001). However, the exact role of host kairomones and/or allomones has not been clearly established (Calderone and Lin, 2001).

The low incidence of mites on queen brood has received less attention. Calderone et al. (2002) found that the rate at which queen brood was infected was independent of the rate of infection of worker brood in the same colony. In addition, eliminating potential worker hosts from the nest had no effect on the incidence of mites in queen cells. However, Santillan-Galicia et al. (2002) and Harizanis (1991) reported that the absence of alternate hosts slightly increased the incidence of infection of queen brood. Trouiller et al. (1994) reported that queen larvae produce about half as much of three putative attractants as worker larvae. They proposed that these differences might account for the low frequency of *V. destructor* on queen brood. Supporting that hypothesis, Calderone et al. (2002) reported that mites in a bioassay were arrested at higher rates by crude extracts of worker larvae than by crude extracts of queen larvae. However, crude extracts of royal jelly (glandular secretions from adult worker bees fed to queen larvae) deterred mites (Calderone et al., 2002), and the larval extracts of the queen larvae may have been contaminated with small quantities of royal jelly, thereby reducing their attractiveness relative to extracts

from worker larvae. This possibility arises from the fact that queen larvae, unlike worker larvae, develop in a pool of royal jelly provided by worker bees in large amounts. In this study, we continue to explore the deterrent activity of royal jelly. We use a bioassay-driven fractionation of a crude extract of royal jelly to identify active fractions and their constituent compounds with deterrent activity.

METHODS AND MATERIALS

Royal Jelly Fractions. Royal jelly was collected from queen cells with larvae between 0 and 12 hr before cell capping. A crude extract of the royal jelly was obtained by extracting it with dichloromethane (four times with 2 ml per gram royal jelly) and then with dichloromethane:MeOH (five times with 1 ml per gram royal jelly) as previously described (Calderone et al., 2002). Bioassay-driven fractionation was performed as outlined in Figure 1.

Step 1: Crude extract equivalent to four gram of fresh royal jelly (4 g RJeq) was applied to a 10.5 × 300 mm open column of 10 g silica gel 60 (70Y230 mesh, E. Merck). In the initial fractionation, hexane:ethyl ether at ratios of 2:1 and 1:1 yielded fractions F1 and F2, and ethyl ether and ethyl ether:MeOH (1:1) were used to elute fractions F3 and F4, respectively. Each fraction was ca. 50 ml.

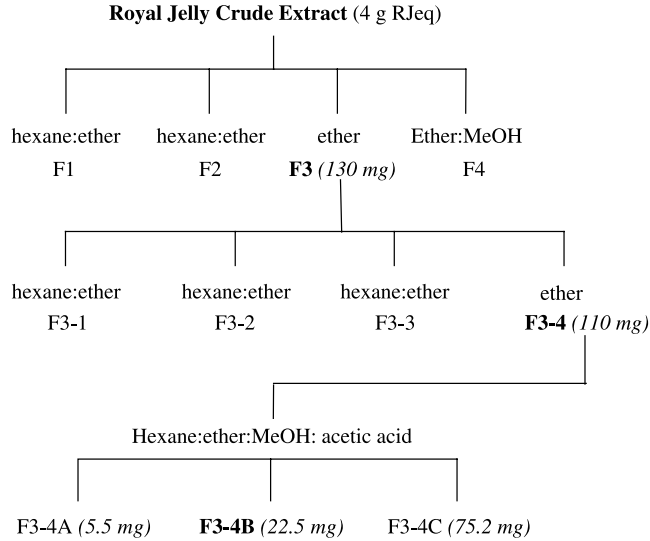

FIG. 1. Scheme for the bioassay-driven fractionation of royal jelly crude extract.

Step 2: Fraction F3, which retained nearly all of the deterrent activity (see below), was further fractionated on an open column of silica gel [10.5 × 300 mm, 10 g silica gel 60 (70Y230 mesh, E. Merck)] with hexane:ethyl ether at ratios 2:1, 1:1, 1:2, and finally with 100% ethyl ether. Of the four resulting fractions (each 45 ml), fraction F3-4 eluted with ethyl ether retained deterrent activity (see below).

Step 3: Fraction F3-4 was further fractionated on an open silica gel column [10.5 × 300 mm, 10 g silica gel 60 (70Y230 mesh, E. Merck)] with hexane:ether:MeOH (10:10:2) and 1% acetic acid as an eluent. Each 4Y5 ml fraction was collected and analyzed with thin-layer chromatography (TLC) using the same eluent. Compounds were visualized using MeOH/sulfuric acid as a charring agent. Three groups of compounds were identified: F3-4A, F3-4B, and F3-4C.

In all steps of the fractionation process, each fraction was concentrated under N_2 to 1 g RJeq per ml CH_2Cl_2 for use in the deterrent contact bioassay. When acetic acid was used in the solvent system, recovered samples were dried under reduced pressure with added water and 2-propanol to remove acetic acid by azeotroping before use in the bioassay.

Chemical Analysis of the Royal Jelly Fractions. Fractions F3-4A, F3-4B,

The reaction was carried out at 50°C for 30 min and directly analyzed with GC and GC-MS.

Chemicals. The following standards were purchased from Sigma-Aldrich Company (St. Louis, MO, USA): 3-hydroxyoctanoic acid, 8-hydroxyoctanoic acid, 3-hydroxydecanoic acid, sebacic acid, 10-hydroxydecanoic acid, 12-hydroxydodecanoic acid, 2-dodecenedioic acid, erucic acid [(*Z*)-13-docosenoic acid], oleic acid [(*Z*)-9-octadecenoic acid], nervonic acid [(*Z*)-15-tetracosenoic acid], (*Z*)-11-eicosenoic acid, (*Z*)-10-heptadecenoic acid, and (*E*)-12-octadecenoic acid.

The (5-1)-hydroxy fatty acids (9-hydroxydecanoic acid, 11-hydroxydodecanoic acid, 13-hydroxytetradecanoic acid, 15-hydroxyhexadecanoic), the (5-2)-hydroxy fatty acids (10-hydroxydodecanoic acid and 12-hydroxytetradecanoic acid), and (*E*)-2-decenedioic acid were synthesized as described below. 10-Hydroxy-(*E*)-2-decenoic acid was available in one of our laboratories (J. Kochansky). All solvents were distilled before use and all chemical reagents were of analytical reagent quality.

Syntheses. The (5-1)- and (5-2)-hydroxy fatty acids were synthesized in two steps. In the first step, an unsaturated fatty acid was ozonized and reduced, forming an oxo-fatty acid. The second step employed a Grignard reaction with an alkyl magnesium bromide.

General Method for the Ozonolysis. Approximately 100 mg of the unsaturated fatty acid [erucic acid, oleic acid, nervonic acid, (*Z*)-11-eicosenoic acid, (*Z*)-10-heptadecenoic acid, or (*E*)-12-octadecenoic acid] was dissolved in 8Y10 ml dichloromethane. A small amount of 2-propanol was added to dissolve the longer fatty acids such as nervonic acid. The solution was stirred at −78°C while ozone was circulated through the reaction vessel until the solution turned blue. Excess ozone was then purged with nitrogen. The ozonide was reduced by adding 2 eq. dimethylsulfide and stirring for 8 hr at room temperature. Thereafter, the solution was concentrated on a rotary evaporator. The mixture was used directly in the Grignard reaction.

General Method for the Grignard Reaction. Ozonolysis products were dissolved in 10 ml dry ether per 100 mg starting material (from the unsaturated fatty acid). The solution was cooled to −30°C and 3Y4 eq. of the Grignard reagent (ca. 0.5Y0.6 ml of a 3 M solution) were slowly added [methyl magnesium bromide to get (5-1)-hydroxy fatty acids and ethyl magnesium bromide to get (5-2)-hydroxy fatty acids] and stirred for 1 hr. After removing the mixture from the dry ice/acetone bath, it was acidified with 1 N HCl to pH 3Y4 and then extracted with ether and dried with anhydrous Na_2SO_4. Solvent was evaporated to yield an oily/solid substance, depending on the hydroxy fatty acid synthesized.

The (5-1)-hydroxy fatty acids were purified with open column chromatography on silica gel [10.5 × 300 mm, 10 g silica gel 60 (70Y230 mesh, E. Merck)] eluting with hexane:ethyl acetate (1:1) and 1% acetic acid. The (5-2)-

hydroxy fatty acids were eluted with hexane:ether (1:1) and 1% acetic acid. Fractions of 4Y5 ml were collected and visualized with TLC. Fractions containing the hydroxy acids were pooled and dried on a rotary evaporator. All hydroxy acids were obtained in >95% purity.

Synthesized compounds were identified by GC/MS and nuclear magnetic resonance (NMR). NMR spectra were recorded at 298 K with a Varian Unity+ (500 MHz proton; 126 MHz carbon) spectrometer. Chemical shifts are reported in parts per million (ppm) relative to the residual $CHCl_3$ peak in solvent $CDCl_3$ at 7.26 ppm unless noted otherwise.

9-Hydroxydecanoic acid: ^{1}H NMR ($CDCl_3$): δ 1.18 (d, 3H, J = 6.2 Hz), 1.25Y1.5 (m, 10H), 1.62 (q, 2H, J = 7.3 Hz), 2.33 (t, 2H, J = 7.5 Hz), 3.8 (sextet, 1H, J = 6.1 Hz). ^{13}C NMR: δ 23.45, 24.84, 25.78, 29.12, 29.33, 29.53, 34.23, 39.27, 68.47, 179.55. MS (as TMS ether methyl ester): *m/z* (%) = 73 (51), 117 (100), 146 (8), 159 (11), 227 (13), 230 (13), 243 (2), 259 (7).

10-Hydroxydodecanoic acid: MS (as TMS ether methyl ester): 73 (65), 131 (100), 146 (6), 159 (11), 169 (14), 244 (11), 255 (14), 273 (46), 287 (2).

11-Hydroxydodecanoic acid: ^{1}H NMR ($CDCl_3$): δ 1.19 (d, 3H, J = 6.2 Hz), 1.23Y1.5 (m, 14H), 1.62 (q, 2H, J = 7.4 Hz), 2.34 (t, 2H, J = 7.5Hz), 3.81 (sextet, 1H, J = 6.0 Hz). ^{13}C NMR: δ 23.49, 24.86, 25.88, 29.12, 29.36, 29.49, 29.65, 29.73, 34.25, 39.36, 68.51, 179.78. MS (as TMS ether methyl ester): 73 (40), 117 (100), 146 (6), 159 (16), 255 (19), 258 (17), 271 (4), 287 (8).

12-Hydroxytetradecanoic acid: ^{1}H NMR ($CDCl_3$): δ 0.94 (t, 3H, J = 7.5 Hz), 1.23Y1.37 (m, 18H), 1.63 (q, 2H, J = 7.6 Hz), 2.34 (t, 2H, J = 7.5Hz), 3.54 (q, 1H, J = 4.7 Hz). ^{13}C NMR: δ 10.09, 24.57, 25.77, 29.17, 29.34, 29.50, 29.58, 29.69, 29.82, 30.23, 34.23, 37.00, 73.67, 179.64. MS (as TMS ether methyl ester): 73 (77), 131 (100), 146 (8), 159 (9), 272 (17), 283 (17), 301 (70).

13-Hydroxytetradecanoic acid: ^{1}H NMR ($CDCl_3$): δ 1.19 (d, 3H, J = 6.2 Hz), 1.22Y1.5 (m, 18H), 1.63 (q, 2H, J = 7.4 Hz), 2.34 (t, 2H, J = 7.5Hz), 3.81 (sextet, 1H, J = 6.0 Hz). ^{13}C NMR: δ 23.56, 24.88, 25.91, 29.20, 29.37, 29.55, 29.66, 29.67, 29.74, 29.77, 34.23, 39.44, 68.53, 179.71. MS (as TMS ether methyl ester): 73 (34), 117 (100), 146 (6), 159 (12), 283 (12), 286 (10), 299 (2), 315 (2).

15-Hydroxyhexadecanoic acid: ^{1}H NMR ($CDCl_3$): δ 1.20 (d, 3H, J = 6.2 Hz), 1.24Y1.35 (m, 22H), 1.64 (q, 2H, J = 7.4 Hz), 2.35 (t, 2H, J = 7.5Hz), 3.81 (sextet, 1H, J = 6.0 Hz). MS (as TMS ether methyl ester): 73 (24), 117 (100), 146 (8), 159 (11), 311 (13), 314 (9), 327 (2), 343 (4).

(E)-2-Decenedioic acid. The intermediate in the synthesis of (*E*)-2-decenedioic acid, 8-oxooctanoic acid, was synthesized from cyclooctene as described by Tolstikov et al. (1982). (*E*)-2-Decenedioic acid was then synthesized from 8-oxooctanoic acid based on a pathway described by Odinokov et al. (1983). A solution of 300 mg 8-oxooctanoic acid, 300 mg malonic acid, and 100 µl piperidine in 2 ml pyridine was kept at room temperature for 30 hr.

Thereafter, it was heated at 110Y115°C for 2 hr, poured into a mixture of 3 ml concentrated HCl in 2.5 g of ice, and extracted three times with 3 ml ether per extraction. The ethereal extract was dried with anhydrous $MgSO_4$. After filtration, the ether was evaporated and a 10% solution of $NaHCO_3$ was added until a pH of 8Y9 was reached. This solution was washed twice with 2 ml ether per wash. The aqueous layer was acidified with 10% HCl to pH 4, then extracted three times with 3 ml ether and dried with anhydrous Na_2SO_4, after which the solvent was evaporated. Purification of the dicarboxylic acid was carried out by recrystallization from hexane and ethyl acetate (1:1) at −20°C, and filtering at this temperature.

(*E*)-*2-Decenedioic acid:* ^{1}H NMR (acetone-d_6): δ 1.35Y1.65 (m, 6H), 2.06 (quintet, 2H, J = 2.5 Hz), 2.24 (dq, 2H, J = 7.5, 1 Hz), 2.3 (t, 2H, J = 7.0 Hz), 5.83 (dt, 1H, J = 15.5, 1.7 Hz), 6.95 (dt, 1H, J =15.4, 6.9 Hz), 10.45 (s, 2H, YOH). MS (as dimethyl ester): 55 (83), 73 (46), 81 (77), 95 (73), 119 (54), 136 (100), 164 (66), 168 (57), 196 (22), 197 (20), 227 (1), 228 (0.1).

Deterrent Bioassay. Deterrent bioassays (Calderone et al., 2002) were conducted in a walk-in environmental chamber maintained at brood nest conditions (32Y34°C and 50% RH). Extracts were presented on a 5.8 × 5.8 cm glass plate with one frosted surface. Glass plates were washed, heated to 175°C for 24 hr, soaked in acetone for at least 12 hr, and rinsed twice with CH_2Cl_2 before each test. A 5 × 5 cm pattern (Figure 2) was printed on paper and attached to the underside of the glass plate to serve as an area marker. An aliquot (2Y10 µl) of extract was applied evenly to the frosted surface in each of the treatment lanes ("T^ in Figure 2) and 10 µl dichloromethane (or ether, as required) were applied for control runs. After air-drying, the plate and pattern were placed on a moistened filter paper in a 9-cm Petri dish. Each bioassay

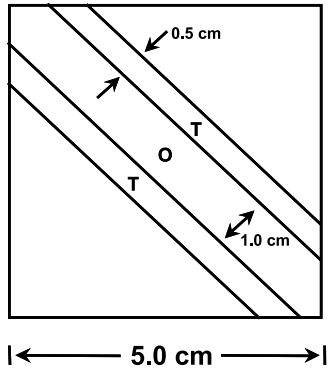

FIG. 2. Diagram of test pattern used to evaluate deterrent activity of extracts in the bioassay. See Methods and Materials section for details.

TABLE 1. CHARACTERISTIC IONS PRODUCED BY FATTY ACID METHYL ESTERS AND TMS ETHERS OF HYDROXY FATTY ACID METHYL ESTERS FOUND IN FRACTION F3-4B AFTER GC/MS ANALYSIS

				Fragments (*m/z*)						Other (*m/z*)								
Peak[a]	Compound	ID[b]	M^+	M-15 ($-CH_3$)	M-29 ($-C_2H_5$)	M-31 ($-CH_3O$)	M-44 ($-C_2H_4O$)	M-47 ($-CH_3 \rightarrow -CH_3OH$)	M-58 ($-C_3H_6O$)	73	89	103	117	131	133	146	159	175
	Diacid																	
6	Sebacic acid (decanedioic acid)[c]	Auth	230			199												
9	Dodecanedioic acid[c]	Auth	258			227												
8	2-Decenedioic acid[c]	Auth	228			197												
17	2-Dodecenedioic acid[c]	Auth	256			225												
	β-Hydroxy acid																	
1	3-Hydroxyoctanoic acid[c]	Auth	246	231		215		199		&	&				&		&	&
4	3-Hydroxydecanoic acid[c]	Auth	274	259		243		227		&	&				&		&	&
	5-Hydroxy acid																	
3	8-Hydroxyoctanoic acid[c]	Auth	246	231		215		199			&	&					&	
7	10-Hydroxydecanoic acid[c]	Auth	274	259		243		227		&	&	&					&	
16	12-Hydroxydodecanoic acid[c]	Auth	302	287		271		255		&		&				&	&	
9	10-Hydroxy-(*E*)-2-decenoic acid[c]	Auth	272	257		241		225		&	&	&						
18	12-Hydroxy-2-dodecenoic acid	Litt	300	285		269		253		&								

	(5-1)-Hydroxy acid													
5	9-Hydroxydecanoic acid[c]	Auth	274	259		243	230	227		&	&		&	&
12	11-Hydroxydodecanoic acid[c]	Auth	302	287		271	258	255		&	&		&	&
20	13-Hydroxytetradecanoic acid[c]	Auth	330	315		299	286	283		&	&		&	&
22	15-Hydroxyhexadecanoic acid[c]	Auth	358	343		327	314	311		&	&		&	&
15	11-Hydroxydodecenoic acid	Litt	300	285		269	256	253		&	&		&	
21	13-Hydroxytetradecenoic acid	Litt	328	313		297	284	281		&	&		&	
	(5-2)-Hydroxy acid													
11	10-Hydroxydodecanoic acid	Auth	302	287	273	271		255	244	&		&		&
19	12-Hydroxytetradecanoic acid[c]	Auth	330		301			283	272	&		&		&
14	10-Hydroxydodecenoic acid	Litt	300	285	271	269		253	242	&		&		

[a] Number corresponds with peak number in Figure 7.
[b] Auth: These compounds were identified by comparison of spectral and chromatographic data of authentic standards. Litt: These were identified according to spectral data from literature or comparing data from analogues (synthesized or commercially available).
[c] Compounds used in the synthetic mixture resembling the F3-4B fraction.

consisted of testing the response of 10Y12 mites to a specific treatment, and each treatment was replicated 2Y12 times, depending on the specific test.

Mites were obtained by using established methods (Kuenen and Calderone, 1997, 1998; Calderone and Lin, 2001). The bioassay was performed by first removing the Petri dish cover, introducing a single mite to the central lane at the point designated "O,^ and replacing the dish cover. Each test period lasted ≤3 min. A finding that a mite would not cross into or through either of the treatment lanes during the test period was used as evidence of a deterrent response. Therefore, a mite was scored as deterred if it moved off the plate while staying within the central lane or if it remained in the central lane for the entire 3-min period, moving and making returns when it contacted either of the treatment lanes. A mite was scored as not deterred if it failed to avoid either of the treatment lanes by moving into or through either of the treatment lanes during the 3-min observation period.

Fractions Evaluated. The royal jelly crude extract was evaluated at three doses (5, 10, and 20 mg RJeq). Fractions F1, F2, F3, and F4 and fractions F3-1, F3-2, F3-3, and F3-4 were each evaluated at 10 mg RJeq. These quantities are smaller than the 200Y300 mg of royal jelly normally available to a queen larva in a queen cell (Schmidt and Buchmann, 1992).

Fractions F3-4A, F3-4B, and F3-4C were each evaluated at three doses: 5, 10, and 20 mg RJeq. The crude royal jelly extract in this test was evaluated at 10 mg RJeq because previous studies (Calderone et al., 2002) have found the response at this level to be equivalent to the response at both 5 and 20 mg RJeq.

A synthetic mixture resembling F3-4B (the most active fraction of the F3-4 fractions) was constituted by combining commercially available standards or synthesized versions of the 15 most prominent compounds identified in that fraction (Table 1). Ether was used as a solvent. Although 10-hydroxydodecanoic acid was synthesized and correctly identified by GC/MS, the yield was very low after purification due to unknown reasons. Therefore, this compound could not be included in the synthetic mixture, and no NMR data could be collected. GC analysis was used to ensure that the quantities and ratios of compounds in the synthetic blend were the same as the corresponding quantities and ratios in the original fraction. The activity of the synthetic mixture (Syn-F3-4B) was compared to that of the native fraction (F3-4B). The amount of the native active fraction (F3-4B) used was 10 mg RJeq. The equivalent dose of the synthetic mixture used was 52 μg/lane. Ether was used as a control.

Statistical Analysis. Arcsine square root transformed data were analyzed using PROC MIXED (SAS, 1996) with fraction modeled as a fixed effect and mite source and the interaction between fraction and mite source as random effects. The TukeyYKramer (SAS, 1988) test was used to compare means.

RESULTS

Royal Jelly Fractions. Bioassay of the royal jelly crude extract at three concentrations (5, 10, and 20 mg RJeq) yielded the same results as previously described by Calderone et al. (2002) (data not shown). There were significant differences among the activities of the four fractions produced in Step 1 of the fractionation process (Figure 3; $F_{4,7} = 30.78$, $P < 0.001$). The deterrent activity of fraction F3 (yield of 130 mg from 4 g RJeq crude extract) was 96.15 ± 0.05%, which was greater than that of the control and the other three fractions (Figure 3; TukeyYKramer, $P < 0.001$ for each significant comparison) and equivalent to the activity observed for the original crude extract (Calderone et al., 2002).

There were significant differences among the activities of the four fractions produced in Step 2 (Figure 4; $F_{4,10} = 15.33$, $P < 0.001$). The deterrent activity of fraction F3-4 (yield of 110 mg) was 89.40 ± 0.08%, which was greater than the activity of the control and the other three fractions (Figure 4; TukeyYKramer, $P < 0.05$ for each significant comparison).

Fraction F3-4 was further fractionated into three fractions: F3-4A (yield of 5.5 mg), F3-4B (22.5 mg), and F3-4C (75.2 mg). Each fraction was evaluated at three doses (5, 10, and 20 mg RJeq). There were significant differences in deterrent activity among these fractions (Figure 5; $F_{10,67} = 18.73$, $P < 0.001$). The activity of each fraction at each dose was greater than the activity of the control (except F3-4A at 5 mg RJeq) (Figure 5; TukeyYKramer, $P < 0.05$ for

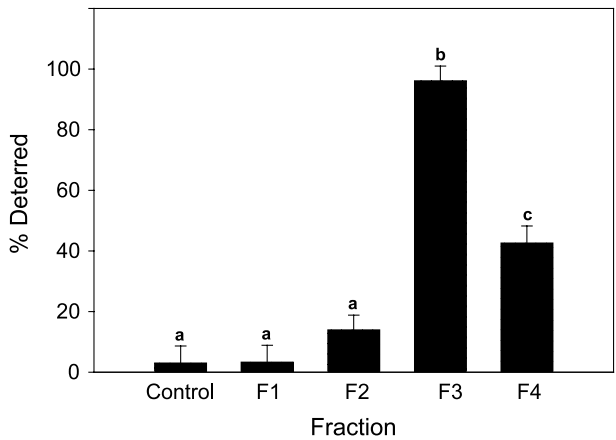

FIG. 3. Deterrent activity of the first four fractions of the royal jelly crude extract produced in Step 1 of the fractionation process. Each fraction was evaluated at 10 mg RJeq. Dichloromethane was used as a control. Bars with different letters are significantly different from each other (TukeyYKramer, $P < 0.05$).

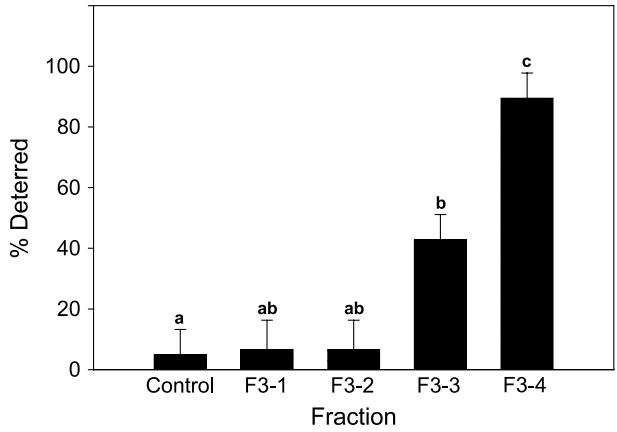

FIG. 4. Deterrent activity of the four fractions derived from fraction F3 in Step 2 of the fractionation process. Each fraction was evaluated at 10 mg RJeq. Dichloromethane was used as a control. Bars with different letters are significantly different from each other (TukeyYKramer, $P < 0.05$).

each significant difference). The activities of fraction F3-4A and F3-4C at each dose were less than that of the crude royal jelly extract (RJ CE) (Figure 5; TukeyYKramer, $P < 0.05$ for each comparison). The activity of F3-4B at each dose tested was equivalent to the activity of the original crude extract (RJ CE) (Figure 5; TukeyYKramer, $P > 0.20$ for each comparison).

There were significant differences in the activities of the original F3-4B fraction, the synthetic F3-4B blend, and the control (Figure 6; $F_{2,40} = 171.19$, $P < 0.001$). The activity of the original F3-4B fraction and the synthetic F3-4B blend were both greater than that of the control (TukeyYKramer, $P < 0.001$ each comparison) but not from each other (TukeyYKramer, $P > 0.75$).

Chemical Analysis. Figure 7 shows the chromatogram of fraction F3-4B analyzed by gas chromatography after methylation and silylation. The compounds present are listed in Table 1. All the compounds identified were fatty acids; more specifically, the extract contained normal fatty acids, diacids, and β-, 5-, (5-1)-, and (5-2)-hydroxy fatty acids. Fraction F3-4A had a similar profile as F3-4B, except that the concentration of the compounds was much lower. The last fraction, F3-4C, was only analyzed on GC. It contained not only some of the compounds found in F3-4B, but also many larger molecules, according to their retention times.

The main reason for derivatization before injecting the samples in the GC was that previous experiments in our laboratory (data not published) as well as earlier studies (Brown et al., 1961; Lercker et al., 1981, and references therein;

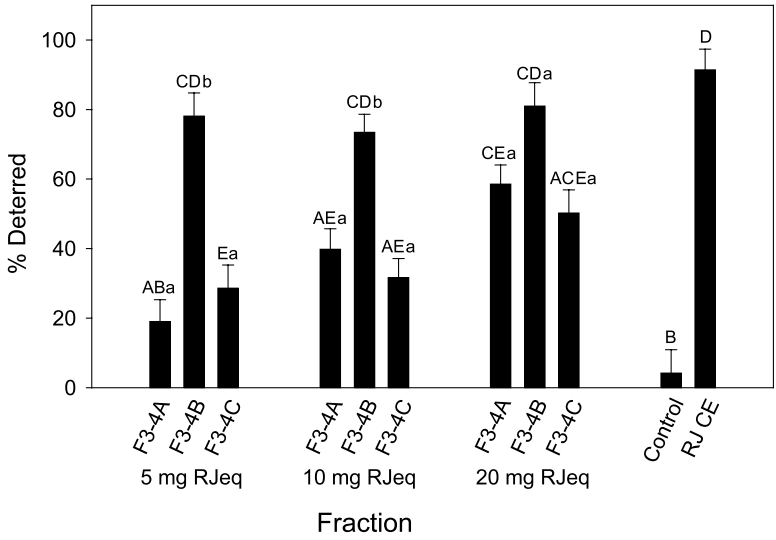

FIG. 5. Deterrent activity of the three fractions derived from fraction F3-4 in Step 3 of the fractionation process. Each fraction was evaluated at three doses: 5, 10, and 20 mg RJeq. Dichloromethane was used as a control and the response to crude extract of royal jelly (RJ) was included for comparison. Results of multiple comparison procedures are shown in lower and uppercase letters. Lowercase letters are valid for comparing responses to the three fractions within a single dose. Uppercase letters are valid for comparing the response of any fractions at one dose with the response to any fractions at the other two doses and to the control and crude extract. Bars with different letters are significantly different from each other (TukeyYKramer, $P < 0.05$).

Lercker et al., 1982) showed that the fractions might contain hydroxy fatty acids. To confirm that these fatty acids were not present as their methyl esters in the original fraction, we carried out an experiment in which the active fraction was silylated with BSTFA without prior methylation. Analysis of this silylated extract revealed that only the TMS esters and TMS ethers of the acids were present; no methyl esters of the fatty acids were found.

The silylated β- and 5-hydroxy fatty acid methyl esters were identified by mass spectrometry according to an extensive study by Eglinton et al. (1968) and Capella and Zorzut (1968). The typical fragments of these fatty acids are given in Table 1. The 5-hydroxy esters all had characteristic peaks at M-15 and M-47 with a smaller peak at M-31. Other peaks present, such as $m/z = 73$ and 89, were all according to Eglinton et al. (1968). The β-hydroxy esters were identified with the same M-15, M-47, and M-31 peaks, but the relative intensities were different from the corresponding ions of the 5-hydroxy esters. Characteristic for

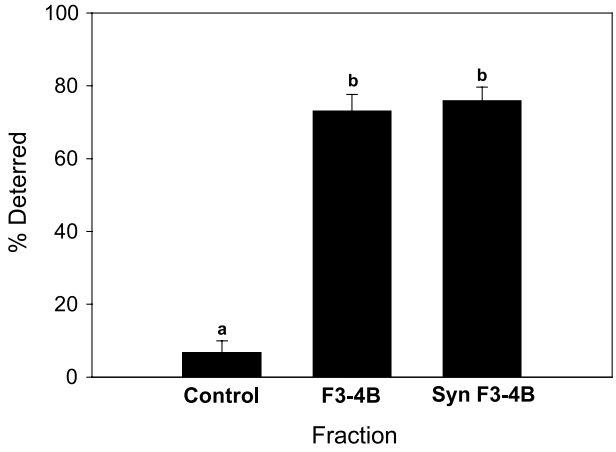

FIG. 6. Deterrent activity of the synthetic blend (Syn-F3-4B) and the original fraction (F3-4B). The amount of the original active fraction (F3-4B) used was 10 mg RJeq. The equivalent dose of the synthetic mixture used was 52 μg/lane. Ether was used as a control. Bars with different letters are significantly different from each other (TukeyYKramer, $P < 0.05$).

these hydroxy esters was the loss of the alkyl chain adjacent to the hydroxy group, resulting in a loss of 56 amu (C_4H_8) for β-hydroxyoctanoic acid and a loss of 84 amu (C_6H_{12}) for β-hydroxydecanoic acid.

All the (5-1)- and (5-2)-hydroxy esters had the M-15, M-47, and M-31 peaks present. In addition to these peaks, an M-44 peak was present in the

(5-1)-hydroxy esters and an M-58 peak was present in the (5-2)-hydroxy esters. This M-44 (or M-58) peak corresponds to a shift of the TMS group from the hydroxy group to the carboxyl group followed by the elimination of $C_xH_{2x}O$. In the (5-2)-hydroxy esters the M-31 peak was small and another peak, M-29 (loss of C_2H_5), was present in this type of ester.

Four unsaturated hydroxy fatty acids, that is, 10-hydroxydodecenoic acid, 11-hydroxydodecenoic acid, 12-hydroxydodecenoic acid, and 13-hydroxytetradecenoic acid, were not synthesized but were tentatively identified by comparison of their mass spectra with those of similar compounds (synthesized or commercially available). Each of these compounds had a similar fragmentation pattern to its analogue. Position and geometry of the double bond were not established in these compounds.

DISCUSSION

Varroa mites were deterred by crude extracts and fractions of crude extracts of royal jelly. Fraction F3-4B exhibited activity equivalent to that of the crude extract, and the activity of the synthetically reconstituted F3-4B fraction was equal to that of the native fraction. The tested amounts of crude extract, active fraction, or synthetically reconstituted fraction were all within the same range and thus comparable to each other. Compared to what a mite would encounter in a queen cell, the amounts used in the bioassay were ca. 10 times

because royal jelly is produced by the mandibular and hypopharyngeal glands of nurse bees. No 9-oxodecanoic acid (9-ODA, or queen substance) was found in the active fraction. Also, other components of the queen mandibular pheromone (QMP), such as the two enantiomers of (*E*)-9-hydroxy-2-decenoic acid, and 4-methyl-3-methoxyphenylethanol were not found. Only a small amount of methyl 4-hydroxybenzoate was found.

The next step in this research will be to determine the contribution of each of the 15 compounds in the synthetic blend to the deterrent activity exhibited by the native fraction. Field tests will be required to provide data on the possible application of these compounds for managing mite levels in honeybee colonies.

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